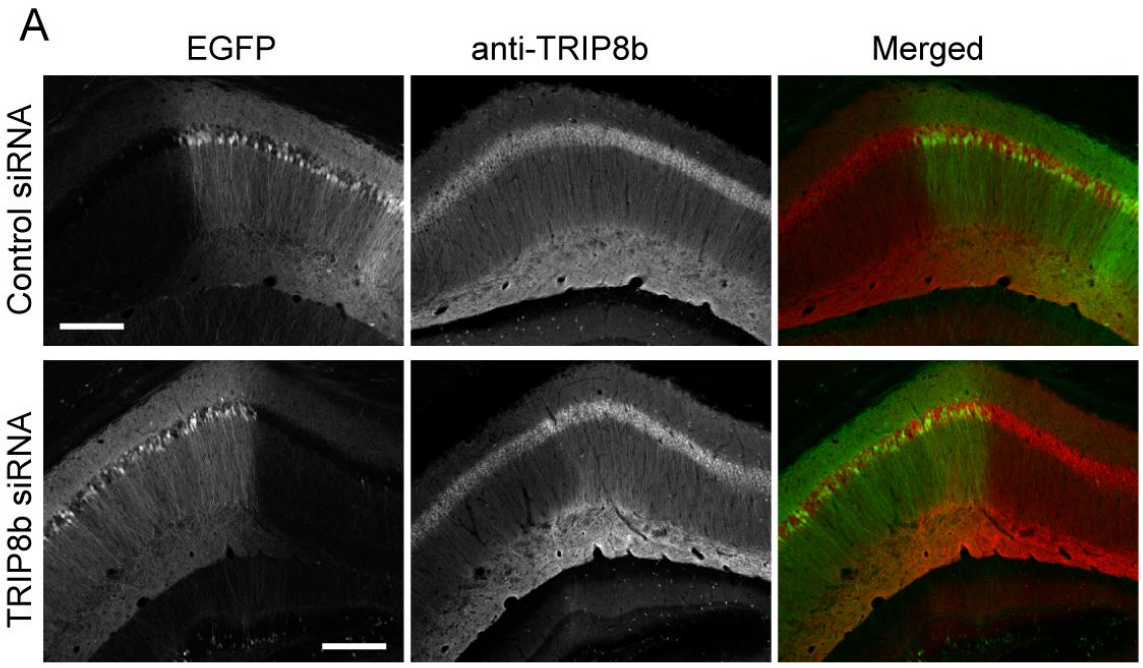
Neuron, Volume *70*

Supplemental Information

TRIP8b Splice Forms Act in Concert to Regulate the Localization and Expression of HCN1 Channels in CA1 Pyramidal Neurons

Rebecca Piskorowski, Bina Santoro, and Steven A. Siegelbaum



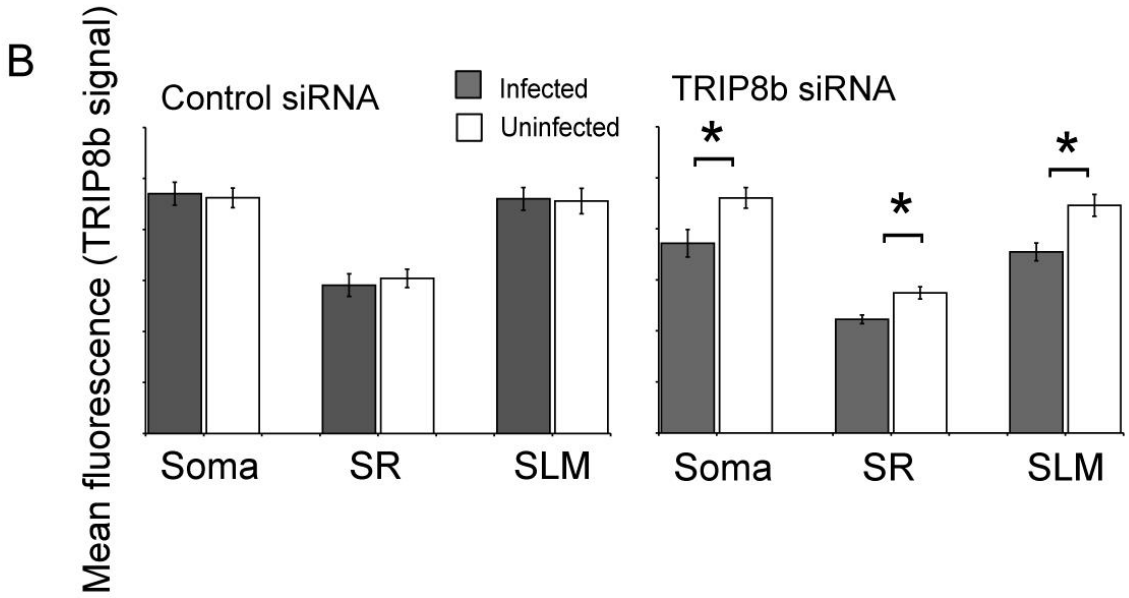
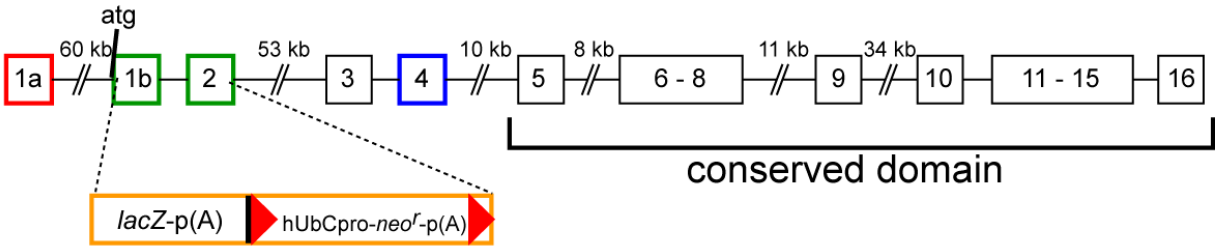


Figure S1, related to figure 1.

Figure S1. TRIP8b siRNA decreased TRIP8b expression *in vivo* as detected by immunohistochemistry. (A) EGFP and TRIP8b expression in hippocampal slices from mice injected with lentiviral vectors expressing EGFP plus either control siRNA (top row) or TRIP8b siRNA (bottom row). Each row is from an independent experiment. Left column: EGFP fluorescence indicating infected region; middle column, immunostaining for TRIP8b; right column, merged image showing EGFP (green) and TRIP8b (red) signals. Note reduction in TRIP8b staining only in region of slices infected with virus expressing TRIP8b knockdown siRNA. (B) Quantification of TRIP8b staining intensity in regions of CA1 that were infected with control (left) or anti-TRIP8b (right) siRNAs. TRIP8b staining intensity was measured in *stratum pyramidale* (SP), *stratum radiatum* (SR) and *stratum lacunosum moleculare* (SLM). Regions that were EGFP positive were compared to regions that were EGFP negative in the same slice. Error bars show SEM. Asterisk denotes significant reduction in TRIP8b staining in region of slices infected with TRIP8b siRNA, relative to both uninfected (EGFP-negative) regions of same slice ($p<0.05$), and regions from a separate population of slices infected with control siRNA. (N=6 mice, 12 injections sites for TRIP8b siRNA; N=4 mice, 8 injections sites for control. Each data point is the average intensity of 17 infected regions).

A TRIP8b 1b/2 KO *(Pex5l$^{tm1(KOMP)Vlcg}$)*

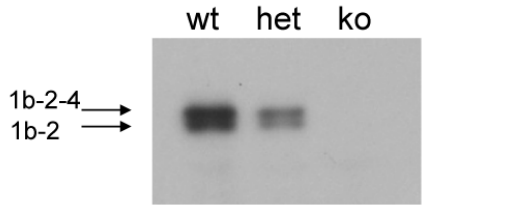


B

TRIP8b Isoform	Effect on HCN1 Expression	% Trip8b mRNA
TRIP8b(1a)	10-fold reduction	25-30%
TRIP8b(1a-2)	no effect	<10%
TRIP8b(1a-2-3-4)	TBD	<1%
TRIP8b(1a-2-4)	5-fold increase	<10%
TRIP8b(1a-3-4)	TBD	<5%
TRIP8b(1a-4)	6-fold increase	30-40%
TRIP8b(1b-2)	>100-fold reduction	10-15%
TRIP8b(1b-2-3-4)	TBD	<1%
TRIP8b(1b-2-4)	>50-fold reduction	10-15%

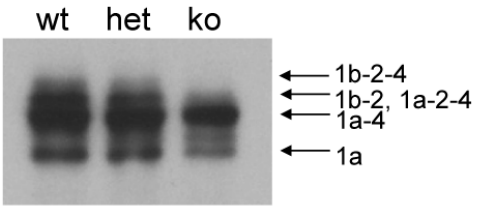
(Santoro et al, 2009)

C



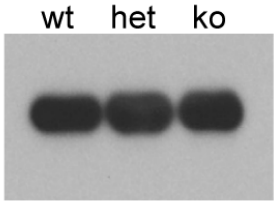
WB:TRIP8b exon 1b ab

E



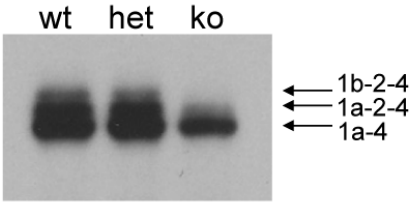
WB:TRIP8b ab (all exons)

D



WB: α-tubulin ab

F



WB:TRIP8b exon 4 ab

Figure S2

Figure S2. Pex5l$^{tm1(KOMP)Vlcg}$, the TRIP8b 1b/2 knockout mouse. (A) Diagram of the TRIP8b gene, showing stategy used by KOMP/Regeneron to replace exons 1b and 2 with the *lacZ* reporter expression cassette and produce the Pex5l$^{tm1(KOMP)Vlcg}$ allele. A second gene expression cassette contained the neomycin resistant gene driven by the human ubiquitin C gene promoter. The neomycin gene is flanked by loxP sites (red arrows). (B) A table listing the previously reported TRIP8b splice isoforms detected in mouse brain mRNA, the effect of each isoform on the surface expression of HCN1 when coexpressed in Xenopus oocytes and the isoform contribution to total brain TRIP8b mRNA (from Santoro et al., 2009). The isoforms shown in green were not expressed in the TRIP8b 1b/2 knockout mouse. (C) Western blot analysis of brain extracts from wild-type, heterozygous and homozygous TRIP8b 1b/2 KO mice showing the dose-dependent loss of TRIP8b(1b-2) and TRIP8b(1b-2-4). A mouse monoclonal antibody recognizing exon 1b (Neuromab) was used to detect TRIP8b(1b-2-4) and TRIP8b(1b-2). (D) Same brain extracts shown in (C) probed with an antibody against α tubulin to control for total loaded protein. (E) Western blot probed with an antibody recognizing all TRIP8b isoforms to detect expression levels of indicated TRIP8b splice forms. Note that TRIP8b(1a-4) and TRIP8b(1a) were still expressed in the TRIP8b 1b/2 knockout brains whereas TRIP8b isoforms containing exons 1b or 2 were not detected. Bands were identified by matching their size to the size of individual TRIP8b isoforms heterologously expressed in *Xenopus* oocytes, as well as by using exon-specific antibodies to immunoprecipitate TRIP8b isoforms from mouse brain extracts, followed by Western blot analysis of the immunoprecipitate with either pan-TRIP8b or complementary exon-specific antibodies (data not shown). (F) Western blot probed with an antibody recognizing TRIP8b exon 4. Note that in extracts from the TRIP8b 1b/2 KO, TRIP8b(1a-4) was still present but TRIP8b(1a-2-4) and TRIP8b(1b-2-4) were absent.

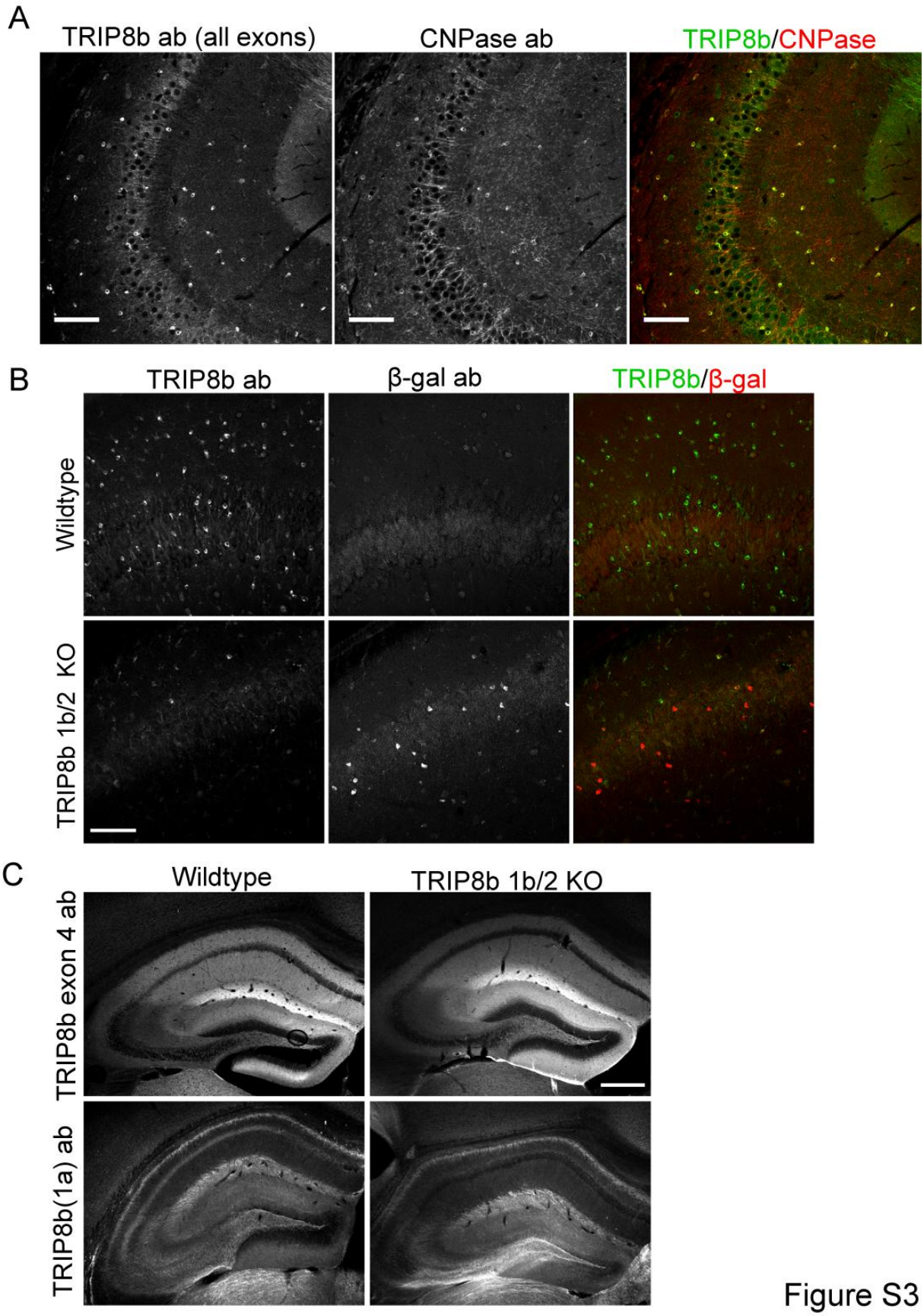

A
TRIP8b ab (all exons)
CNPase ab
TRIP8b/CNPase
B
TRIP8b ab
β-gal ab
TRIP8b/β-gal
Wildtype
TRIP8b 1b/2 KO
C
Wildtype
TRIP8b 1b/2 KO
TRIP8b exon 4 ab
TRIP8b(1a) ab

Figure S3

Figure S3. TRIP8b isoforms containing exons 1b or 2 are not expressed in pyramidal cells, but are present in oligodendrocytes. (A) Immunfluorescence with antibody that detects all TRIP8b isoforms labelled very small cells in the hippocampal CA3 region (left and green signal on right). These small cells were also labeled with an antibody to CNPase (center and red signal on right), an oligodendrocyte marker. Scale bar 100 μm. (B) TRIP8b antibody staining of small cells in CA3 was lost in the TRIP8b 1b/2 KO animals. Top row, Immunfluorescence for wild-type littermate controls. Left panel, Staining with pan-TRIP8b antibody. Center panel, Staining with antibody to β-galactosidase. Right, Antibody staining for TRIP8b (green) and β-galactosidase (red). Bottom row, Immunfluorescence for TRIP8b exon 1b/2 KO mice. Panels show staining with same antibodies as in top row. Scale bar 100 μm. (C) Immunofluorescence with antibodies specific for either TRIP8b exon 4 (top row) or TRIP8b(1a) (bottom row) for hippocampal slices from wild-type and 1b/2 KO mice. Scale bar, 500 μm.

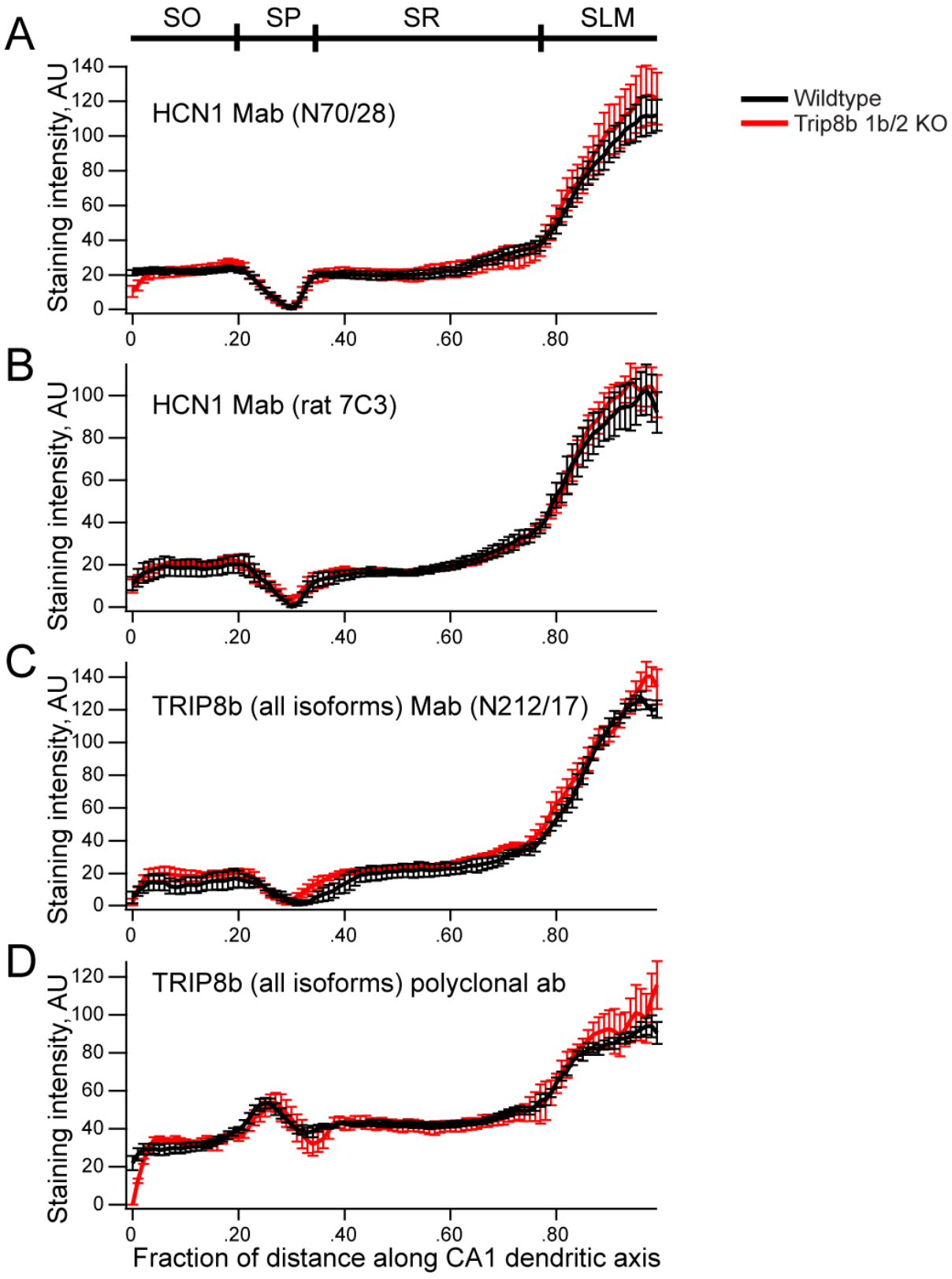


Figure S4, related to Figure 6

Figure S4. Quantification of HCN1 and TRIP8b CA1 dendritic gradients in wild-type and TRIP8b 1b/2 KO mice. For all panels, the fluorescence intensity from coronal hippocampal slices immunostained with four different antibodies is plotted as a function of distance along the CA1 axis. The different dendritic layers, SO, SP, SR and SLM, are indicated at the top. Red traces correspond to TRIP8b 1b/2 KO mice and black to wildtype littermate controls. (A) Immunolabeling performed with an HCN1 monoclonal ab (N70/28). (B) Immunolabeling performed with a different HCN1 monoclonal ab (recognizes a different epitope from (A), rat 7C3). (C) Immunolabeling with a monoclonal ab that recognizes all TRIP8b splice isoforms (N212/17). (D) Immunlabeling with a polyclonal antibody that recognizes all TRIP8b splice isoforms (794). For all panels, N= 2 animals for both TRIP8b 1b/2 KO and control. 3 slices were analyzed from each animal. Plots show mean ± SEM.

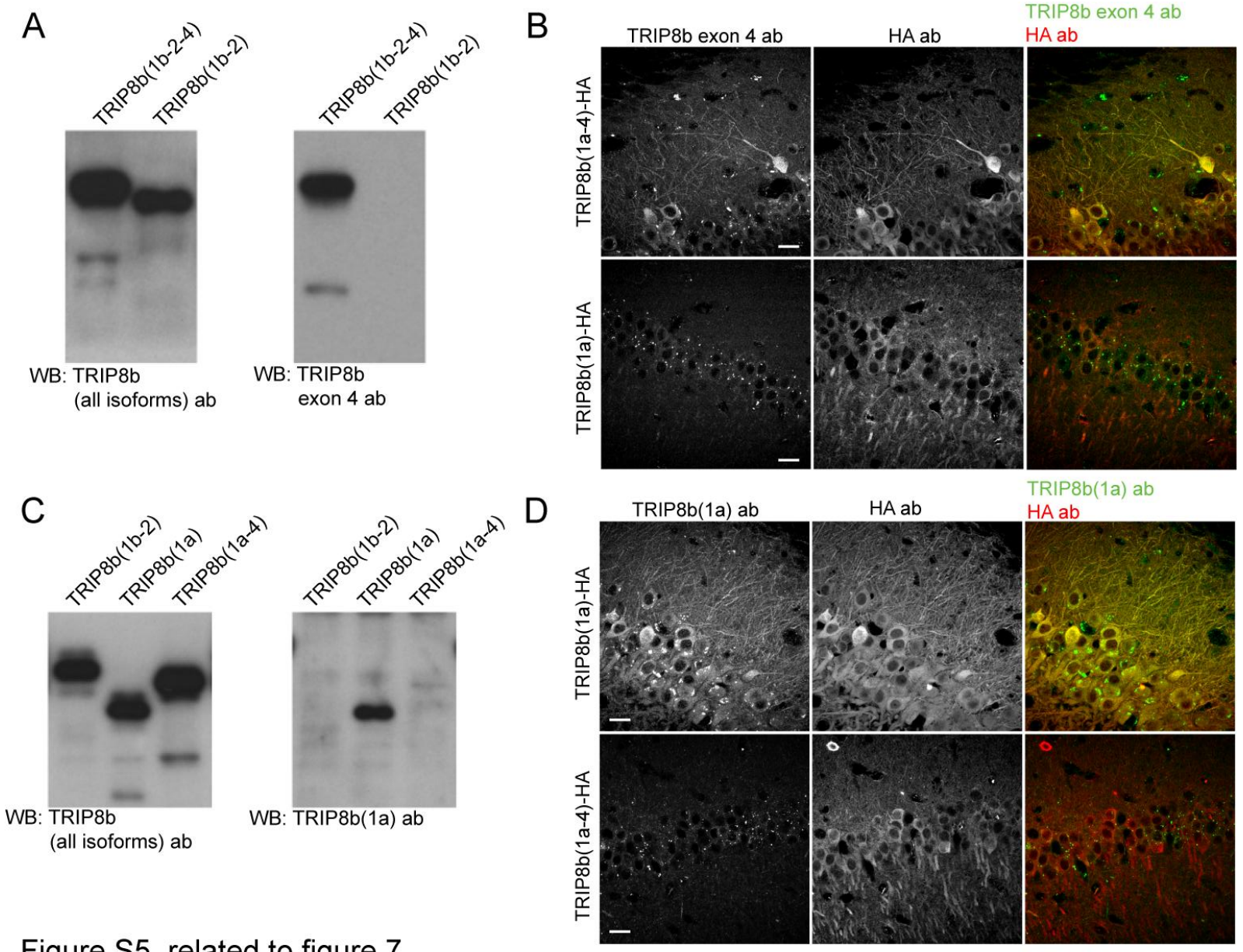


Figure S5, related to figure 7

Figure S5. Specificity of TRIP8b(1a-4) and TRIP8b(1a) antibodies. (A) Western blot analysis of *Xenopus* oocyte extracts that had been injected with cRNA encoding TRIP8b(1b-2-4) or TRIP8b(1b-2). Left, blots probed with TRIP8b antibody that recognizes all isoforms. Right, same extracts probed with a mouse monoclonal antibody that recognizes TRIP8b exon 4. Note that TRIP8b(1b-2-4) is recognized whereas TRIP8b(1b-2) is not**.** (B) Immunofluorescence of hippocampal slices from mice injected in CA1 with lentivirus expressing TRIP8b(1a-4)-HA (top row) or TRIP8b(1a)-HA (bottom row). Left column, slices stained with exon 4 antibody. Middle column, slices stained with HA antibody. Right column, superposition of exon 4 (green) and HA (red) staining. Note: co-labeling between exon 4 and HA antibodies was observed in neurons infected with TRIP8b(1a-4)-HA but not TRIP8b(1a)-HA. Scale bar 20 μm. (C) Western blots from extracts of *Xenopus* oocytes expressing TRIP8b(1b-2), TRIP8b(1a) and TRIP8b(1a-4). Left, blots probed with TRIP8b antibody that recognizes all isoforms. Right, same extracts probed with TRIP8b(1a) antibody. (D) Immunofluorescence from similar experiment as shown in (B) showing neurons virally expressing TRIP8b(1a)-HA (top row) were co-labeled with TRIP8b(1a) (left column) and HA (center column) antibodies. Neurons virally expressing TRIP8b(1a-4)-HA were not co-labeled (bottom row). Scale bar, 20 μm.

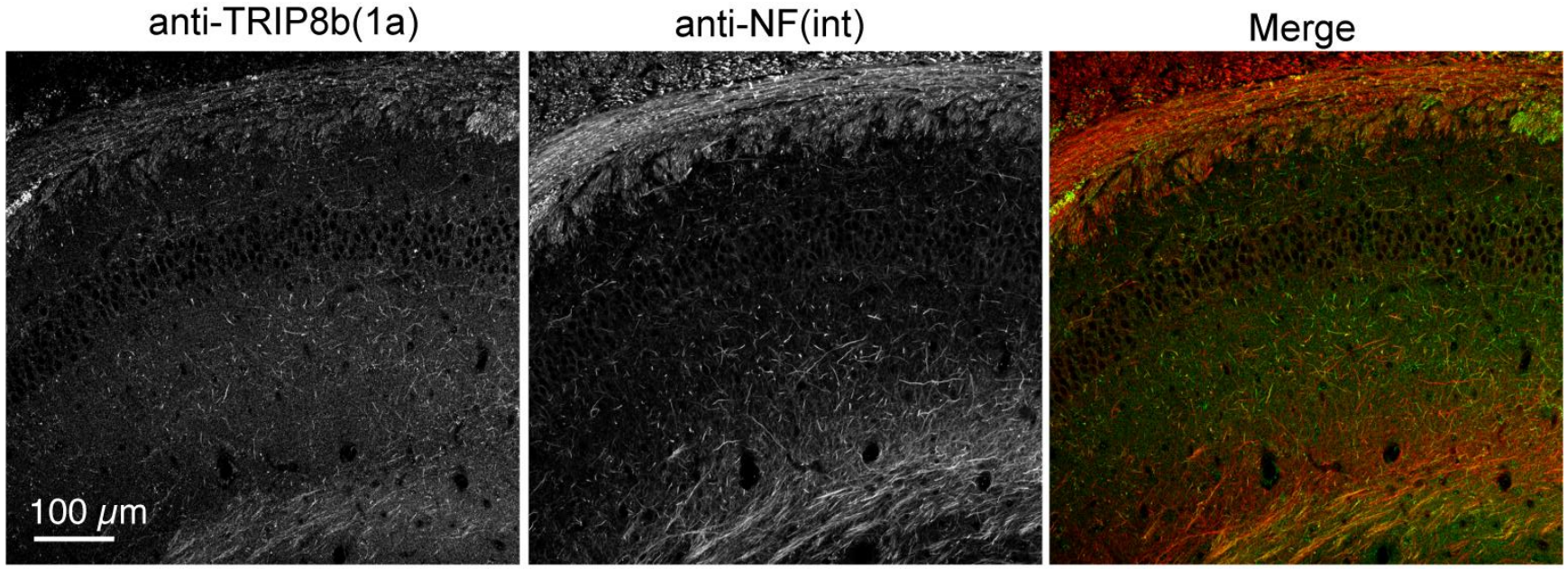


Figure S6

Figure S6. Fibers labeled by TRIP8b(1a) antibody are also labeled with antibody to intermediate sized neurofilament. (A) Left, Staining of the CA1 region of a hippocampal slice by a chicken polyclonal antibody to TRIP8b(1a). Center, Staining of CA1 region of same slice with antibody to intermediate-sized neurofilament, NF(int), an axonal marker. All fibers labeled with the TRIP8b(1a) antibody are colabeled with the antibody to NF(int), indicating axonal localization of TRIP8b(1a).

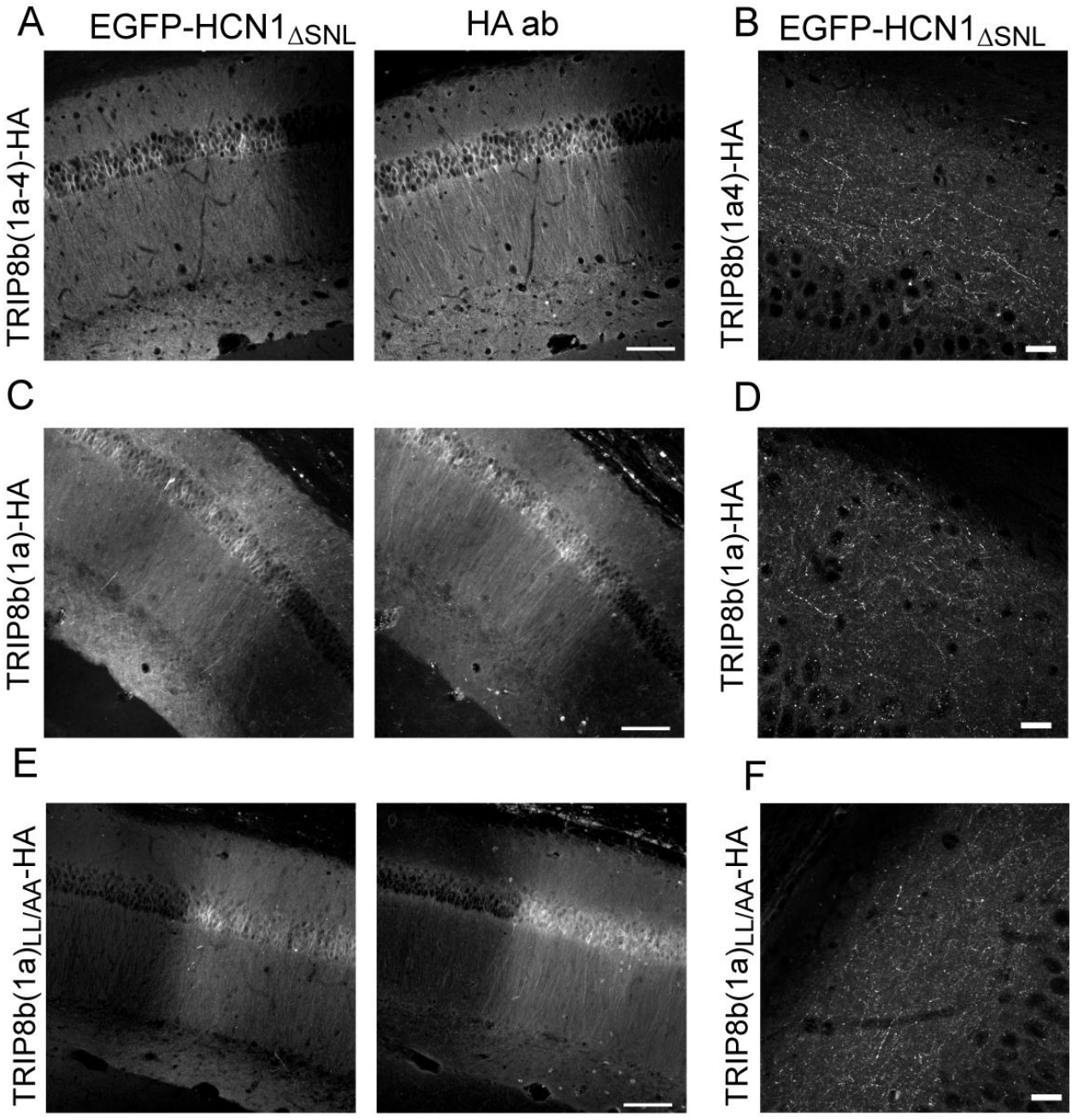


Figure S7, Related to figure 8

Figure S7. Co-expression of HA-tagged TRIP8b splice forms has no effect on the expression pattern of EGFP-HCN1$_{\Delta SNL}$. Confocal z-series of pyramidal neurons in the CA1 region of the hippocampus that have been co-infected with EGFP-HCN1$_{\Delta SNL}$ and HA-tagged TRIP8b isoforms. (A, C, E) Left panels, EGFP-HCN1$_{\Delta SNL}$ fluorescence images in response to viral expression of TRIP8b(1a-4) (A), TRIP8b(1a) (B), or TRIP8b(1a)$_{LL/AA}$ (C). Right panels, HA antibody signal detecting virally expressed HA-tagged TRIP8b isoforms. Scale bar 100 μm. (B, D, F) Higher-magnification z-series projection images of EGFP signal from hippocampus contralateral to that coinjected with virus expressing EGFP-HCN1$_{\Delta SNL}$ with TRIP8b(1a-4) (B), TRIP8b(1a) (D) or TRIP8b(1a)$_{LL/AA}$ (F). Scale bar 20 μm. Note that over-expression of TRIP8b splice forms has no effect on EGFP-HCN1$_{\Delta SNL}$ distribution (compare to Figure 4 showing EGFP-HCN1$_{\Delta SNL}$ signal without TRIP8b coexpression).

Supplemental Experimental Procedures

Lentivirus expression. The lentiviral expression vector containing the CaMKII promoter, pFCK(0.4)GW was kindly provided by Pavel Osten (Max Planck Institute, Heidelberg; Dittgen et al., 2004). Each lentiviral expression plasmid was transfected into HEK293FT cells with virus packaging plasmids pVSVg and pΔ8.9. After 24-48 hours the culture media containing assembled viruses was filtered, placed over a 20% sucrose cushion and spun at 26000 rpm for 3 h to purify the virus. The pellet was re-suspended in sterile saline and stored at -80 ºC. Virus titers were measured using high-density cultured hippocampal neurons. For *in vivo* delivery, virus was concentrated to 10^8 IU/ml in sterile saline and stereotaxically injected into the hippocampal CA1 region of adult mice (age 3-9 months). HCN1 knockout animals (genotype $HCN1^{-/-}$) were generated and bred as described (Nolan et al., 2003).

siRNA knock-down. siRNA target sequences were selected using the GenScript and Ambion algorithms. The searches were run using exclusively the constant region of the TRIP8b open reading frame (exons 5-16 of the mouse cDNA sequence). siRNA target sequences identified by both algorithms were cloned into the pLLhS lentivirus vector (Nakagawa et al., 2004), under the control of the U6 promoter, by designing the corresponding dsDNA oligonucleotides. Specifically, for the siRNA used in the experiments shown in the present study, the following oligos were used:

GS2 forward

TGTTGAACTCTCCACTCAGGTGGTTCAAGAGACCACCTGAGTGGAGAGTTCAATTTTTTC

and GS2 reverse

TCGAGAAAAAATTGAACTCTCCACTCAGGTGGTCTCTTGAACCACCTGAGTGGAGAGTTCA

ACA

Oligos were annealed, and inserted between the HpaI and XhoI sites of the pLLhS vector. Note that in order to test that the activity of our chosen siRNA was similar to what was previously described (Lewis et al., 2009), and thus confirm the effects seen on the Ih conductance through

two independent siRNA sequences, we recloned and expressed the TRIP8b-sh4 siRNA sequence used by Lewis et al (2009) into the pLLhS vector. The control siRNA construct was similarly constructed using vector pLLhS, but encodes a scrambled target sequence.

siRNA efficacy was assayed by Western blot analysis from cultured neuronal extracts 7 days after infection with virus. The anti-TRIP8b siRNA chosen for the present study was selected based on the observation that it led to the largest reduction in the levels of TRIP8b protein (Figure 1A) as compared to a control siRNA. This TRIP8b-specific siRNA construct targeted the sequence 5'-CCACCTGAGTGGAGAGTTCAA-3' corresponding to nucleotide positions 1419-1439 in the TRIP8b(1b-2) isoform cDNA sequence, which is in exon 14 (Santoro et al., 2009). We ensured that the effects of the knockdown that we observed in culture with our constructs was equivalent to the knockdown previously reported with other constructs (Lewis et al., 2009) (data not shown). Whole-cell current and voltage clamp recordings obtained 7-10 days after infection demonstrated a marked reduction of Ih in cultured neurons expressing the anti-TRIP8b siRNA compared to neurons expressing control siRNA.

Antibodies. Antibodies used for immunohistochemistry and/or Western blot analysis were: rat monoclonal anti-HCN1 (Muller et al., 2003; clone 7C3) mouse monoclonal anti-HCN1 (Neuromab, clone N70/28) rabbit anti-TRIP8b (Santoro et al., 2009, 794), mouse monoclonal anti-TRIP8b (Neuromab, clone N212/17), mouse monoclonal anti-TRIP8b exon 4 (Neuromab, clone N212/3), mouse monoclonal anti-TRIP8b exon 1b (Neuromab, clone N212/32), chicken polyclonal anti-MAP2 (abCam), rat monoclonal anti-HA (Roche, clone 3F10), mouse monoclonal int-NF (Calbiochem), mouse monoclonal anti-CNPase (Sigma), and chicken polyclonal anti-β-galactosidase (abCam). The polyclonal anti-TRIP8b(1a) antibody was raised in chicken against a peptide spanning the junction of TRIP8b exons 1a and 5 (sequence MYQGHMQLVNEQKKC). Sera were screened and specificity tested as shown in Supplemental Figure S5. Secondary antibodies for fluorescence immunohistochemistry were: rhodamine-redX conjugated goat anti-rat, goat anti-chicken, Cy5-conjugated goat anti-rabbit and goat anti-rat

(Jackson ImmunoResearch); alexa568-conjugated goat anti-mouse and alexa568-conjugated goat anti-chicken (Molecular Probes).

Hippocampal cultures and electrophysiology. The hippocampus was dissected from the brains of P0 rats, digested with papain, washed with protease inhibitor, then triturated. Neurons were counted, plated onto differentiated primary astrocyte feeder layers, and grown in Neurobasal media supplemented with B27, glutamine and antibiotics. Cultures were infected with lentivirus expressing either control siRNA or TRIP8b siRNA, both under the control of an ubiquitin promoter, together with EGFP under control of an independent synapsin promoter.

Whole-cell patch recordings were obtained from pyramidal-shaped neurons 7-11 days after infection using 2-3 MΩ borosilicate glass pipettes. Intracellular solution contained (in mM): KMethylsulfate (130), KCl (10), HEPES (10), NaCl(4), EGTA (0.1), MgATP (4), Na_2GTP (0.3), Phosphocreatine (10). The external bath solution contained (in mM): 145 NaCl, 3 KCl, 10 HEPES, 3 $CaCl_2$, 8 glucose, 2 $MgCl_2$ (pH adjusted to 7.30 with NaOH). All recordings were performed at 31° C. Series resistance was less than 15 MΩ, which was then compensated by 80-90%. To eliminate spontaneous activity, 2 µM tetrodotoxin (TTX) was added to the bath solution after establishing that a cell displayed the expected action potential shape and firing pattern for a pyramidal neuron. Ih current density was measured by holding the cells at -70 mV and proceeding through a series of 3-s voltage steps to potentials from -40 to -130 mV, followed by a voltage step to -120 mV to measure the tail current. The tail current I-V curve was fit with a Boltzmann function, I(V) = A1 + A2/(1 + exp[(V-V1/2)/s]), in which A1 is the offset caused by holding current, A2 is the maximal tail current amplitude, V is the test pulse voltage, $V_{1/2}$ is the midpoint voltage of activation, and s is the slope (in mV). Ih current density was calculated by dividing the maximal tail current (A2) by the cell capacitance. All data were obtained in pairs, with one control siRNA expressing neuron matched with each TRIP8b siRNA expressing neuron, as described.

Acute hippocampal slices and electrophysiology. Slices were cut on a LeicaVT1000S vibratome in cold (3-4 ºC) dissection solution containing (in mM) 10 NaCl, 1.25 NaH_2PO_4, 2.5 KCl, 25 $NaHCO_3$, 25 glucose, 0.5 $CaCl_2$, 7 $MgCl_2$, 190 sucrose, and 2 Na-pyruvate, equilibrated with 95%/5% O_2/CO_2. All recordings were performed in standard ACSF solution containing 125 NaCl, 1.25 NaH_2PO_4, 2.5 KCl, 25 $NaHCO_3$, 25 glucose, 2 $CaCl_2$, 1 $MgCl_2$, and 2 Na-pyruvate equilibrated with 95%/5% O_2/CO_2. All recordings were performed at 31° C. In all experiments, inhibitory transmission was blocked by the $GABA_A$ and $GABA_B$ receptor antagonists: 1 µM gabazine and 2µM CGP-55845, and an incision was made between CA1 and CA3 to prevent epileptic activity. PP and SC inputs were stimulated with focal stimulating pipettes filled with 1M NaCl and placed approximately 200 µm away from the recording pipette. Stimulus intensity was adjusted as previously described (Nolan et al., 2004). Whole cell recordings were obtained from CA1 pyramidal cells visually identified using DIC optics. Input resistance was determined by injecting a small hyperpolarizing current step of –50 pA for 500 ms from the resting potential and dividing the change in steady-state voltage (ΔVss) by the injected current. Hyperpolarizing current injections were used to measure the sag. The sag ratio was measured as (1 – ΔVss/ΔVmin) x 100%, where ΔVss is the steady-state hyperpolarization (relative to resting potential) at the end of the hyperpolarizing pulse and ΔVmin is the peak hyperpolarization near the beginning of the current step. The amplitude of the current step was adjusted so that a constant value of Vmin (between –90 and –95 mV) was achieved, to assure uniform activation of Ih during the hyperpolarization in different cells and under different conditions. Patch pipettes (3-5 MΩ) were filled with intracellular solution containing (in mM) 135 $KMeSO_4$, 5 KCl, 0.1 EGTA, 10 HEPES, 2 NaCl, 5 MgATP, 0.4 Na_2GTP, 10 phosphocreatine, and 10 µM Alexafluor 594 cadaverine.

Supplemental Material References:

Muller, F., Scholten, A., Ivanova, E., Haverkamp, S., Kremmer, E., and Kaupp, U.B. (2003). HCN channels are expressed differentially in retinal bipolar cells and concentrated at synaptic terminals. Eur J Neurosci *17*, 2084-2096.